Progress in Neuro-Psychopharmacology & Biological Psychiatry 76 (2017) 169–178

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Oxytocin mitigated the depressive-like behaviors of maternal separation stress through modulating mitochondrial function and neuroinflammation

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ARTICLE INFO

Article history:
Received 26 September 2016
Received in revised form 25 February 2017
Accepted 28 February 2017
Available online 01 March 2017

Keywords:
Maternal separation stress
Depression
Oxytocin
Neuroinflammation
Mitochondrial function

ABSTRACT

Mother-infant contact has a critical role on brain development and behavior. Experiencing early-life adversities (such as maternal separation stress or MS in rodents) results in adaptations of neurotransmission systems, which may subsequently increase the risk of depression symptoms later in life. In this study, we show that Oxytocin (OT) exerted antioxidant and anti-inflammatory properties. Previous studies indicate that neuroinflammation and mitochondrial dysfunction are associated with the pathophysiology of depression. To investigate the antidepressant-like effects of OT, we applied MS paradigm (as a valid animal model of depression) to male mice at postnatal day (PND) 2 to PND 14 (3 h daily, 9 AM to 12 AM) and investigated the depressive-like behaviors of these animals at PND 60 in different groups. Animals in this work were divided into 4 experimental groups: 1) saline-treated, 2) OT-treated, 3) atosiban (OT antagonist)-treated and, 4) OT + atosiban-treated mice. We used forced swimming test (FST), splash test, sucrose preference test (SPT) and open field test (OFT) for behavioral assessment. Additionally, we used another set of animals to investigate the effects of MS and different treatments on mitochondrial function and the expression of the relevant genes for neuroinflammation. Our results showed that MS provoked depressive- like behaviors in the FST, SPT and splash test. In addition, our molecular findings revealed that MS is capable of inducing abnormal mitochondrial function and immune-inflammatory response in the hippocampus. Further, we observed that treating stressed animals with OT (intracerebroventricular, i.c.v. injection) attenuated the MS-induced depressive-like behaviors through improving mitochondrial function and decreasing the hippocampal expression of immune-inflammatory genes. In conclusion, we showed that MS-induced depressive-like behaviors in adult male mice are associated with abnormal mitochondrial function and immune-inflammatory responses in the hippocampus, and activation of OTergic system has protective effects against negative effects of MS on brain and behavior of animals.

1. Introduction

Mother-infant contact has an important effect on the shaping of newborns' physiological system along with the development/maturation of the brain and behavior. In this regard, it has been shown that

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http://dx.doi.org/10.1016/j.pnpbp.2017.02.022
0278-5846/

early life maternal care is linked with bio-behavioral improvements which contributes to the shaping of behavioral and physiological progressions, and subsequently increasing social adaptation (Feldman et al., 2010). Research studies indicate that early life exposure to stressors and traumatic events increase the risk for developing neuropsychiatric disorders in later life (Diekstra and Wolters, 1992; Heim and Nemeroff, 2001; Johnson et al., 2002; Millstein and Holmes, 2007; Kendler et al., 2010). In rodents, early life maternal separation stress (as a valid animal model of depression) negatively affects different brain areas such as amygdala, prefrontal cortex and hippocampus and alters neurotransmission (Millstein and Holmes, 2007; Amini-Khoei et al., 2015; Amiri et al., 2016a). These bio-behavioral and neurochemical alterations are associated with the development of a variety of neuropsychiatric disorders such as depression in both human and animals in adulthood (Zlotnick et al., 1995; Brodsky et al., 2001; Roy, 2002; Diehl et al., 2012). Additionally, research evidence has shown that early life stress is capable of altering normal mitochondrial function in the brain by affecting the antioxidant system and overproduction of free radicals, including reactive oxygen species (ROS) (Diehl et al., 2012; Hendricks et al., 2012). ROS production Oxidative stress is suggested to be produced due to an imbalance between oxidative and antioxidative system, and plays an important role in the pathogenesis of a wide range of neuropsychiatric diseases such as depression (Sarandol et al., 2007; Pandya et al., 2013; Zhang and Yao, 2013). Depression is considered as a mental disorder which is accompanied by oxidative damage and a significant impairment in antioxidant system (Maurya et al., 2016). Ample evidence suggests that mitochondria are among the main targets of stress, and abnormal mitochondrial activity is associated with the initiation of immune-inflammatory responses in the brain (Hagberg et al., 2014; Picard et al., 2014).

On the other hand, neuroinflammation is a major contributing factor in the pathophysiology of psychiatric disorders such as depression (Hurley and Tizabi, 2013). It has been shown that chronic exposure to stress triggers the immune-inflammatory responses in the brain, and induces depressive-like behaviors in animals (Frank et al., 2013).

Oxytocin, a nonapeptide neuromodulator, plays a crucial role in the regulation of mammalian social behaviors, stress responses, emotions and reproductive behaviors (Higuchi, 1995; Boccia et al., 2013; Love, 2014; Shamay-Tsoory and Young, 2016). Recent investigations on the OTergic system have been shown that this system could be considered as a potential pharmacologic target in psychiatric disorders that can improve some symptoms of social dysfunctions in autism, schizophrenia, social anxiety and borderline personality disorder (Shamay-Tsoory and Abu-Akel, 2016). Recently, OTergic system has gained the attention of researchers for its antidepressant properties (McQuaid et al., 2014).

Although there is evidence indicating the association between mitochondrial dysfunction and neuroinflammation (Hagberg et al., 2014; Picard et al., 2014; Möller et al., 2015), but few studies have focused to investigate whether MS stress is accompanied by immune-inflammatory responses. Further, considering the beneficial effects of OT as an pharmacological agent with anti-inflammatory and antioxidant properties (Karelina et al., 2011), we aimed to investigate the effects of exogenous OT on the depressive-like behaviors in an animal model of early life stress considering the role of immune-inflammatory responses and mitochondrial function.

2. Materials and methods

2.1. Animals and housing conditions

Pregnant NMRI mice (gestation day 1) were purchased from Pasteur Institute of Iran, and used in current study. Animals were maintained under standard laboratory conditions as 12-h light/dark cycle, temperature 22 ± 1 °C and free access to food and water ad libitum. The birth date was considered as postnatal day 0 (PND 0). After birth, at PND 2 the offsprings were subjected to MS paradigm. For this purpose, pups were separated from their mothers for 3 h daily during PND 2-14, beginning at 09:00 a.m., and were returned to their mothers after the 3 h separation period (Desbonnet et al., 2010; Amini-Khoei et al., 2015; Amiri et al., 2016a). At the end of PND 14, pups were returned to their mothers cages and remained undisturbed till PND 21. On PND 21, male mice were weaned and kept in groups (4 mice per cage) until experiment day PND 60. We did not touch the control animals and they left undisturbed and were weaned on PND 21 and grouped in cages (n = 4) till PND 60 for experiments.

All procedures in this study were done in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) and institutional guidelines for animal care and use (Department of Pharmacology, Faculty of Medicine, Tehran University of Medical Sciences). All tests were performed between 10:00 a.m. to 02:00 p.m. All experimental groups involved 3–8 mice and we tried to minimize the use of animals and to improve their well-being.

2.2. Study design

This research study was carried out in two different parts. For the first part, we applied OT at different doses (0.5, 0.75, 1 and 1.25 μg/mouse in 1 μl) to treat both control and MS mice in order to find the effective and sub-threshold doses of OT. On the second part, both MS and control mice were subjected to behavioral tests including forced swimming test (FST), open-field test (OFT), splash test and sucrose preference test (SPT). The treatment as primary factor was assigned randomly to both control and MS mice as follows 1) saline, 2) OT (1 μg/mouse in 1 μl, 30 min before test), 3) atosiban, a specific antagonist of OT receptor, (10 μg/mouse in 1 μl, 15 min before test), and 4) atosiban + OT (atosiban 15 min prior to OT injection).

All behavioral experiments were performed in adult mice (PND 60-62). At the end of our study, the animals were sacrificed (decapitated) and the murine hippocampi were separated on ice-cold surface and directly snapped freeze in liquid nitrogen and kept in freezer −80 °C until the start of molecular assays. Above-mentioned drugs were injected intracerebroventricularly (i.c.v.) according to the method described by Haley and McCormick (Haley and McCormick, 1957). The doses and time of drug administrations were selected according to the previous studies (Qi et al., 2012; Han et al., 2014; Amini-Khoei et al., 2016) and our pilot studies.

2.3. Behavioral experiments

2.3.1. Forced swimming test (FST)

In the FST, the increased immobility time is associated with despair behavior reflecting the depressive-like behavior in rodents (Porsolt et al., 1977; Haj-Mirzaian et al., 2016a). Mice were separately placed in an open glass cylinder (diameter: 10 cm, height: 25 cm) filled with 19 cm water (23 ± 1 °C). Mice were allowed to swim for a 6 min period and the immobility time was recorded during the last 4 min of the test by an experimenter

blinded to the treatment or environment conditions. Immobility time was considered when the mouse remained immobile in the water and made only those essential movements to maintain its head above the water.

2.3.2. Open-field test (OFT)

We used OFT to investigate the effects of housing condition and treatments on motor function (Kulesskaya and Voikar, 2014). The OFT device was made of white opaque Plexiglas (50 cm × 50 cm × 30 cm) which was dimly illuminated. Each animal was gently placed on the central zone of the apparatus (30 cm × 30 cm) and its behavior was recorded using a camera for 5 min and evaluated by Ethovision software version 8 (Noldus, Netherlands). The distance moved (horizontal activity) and the number of rearings (vertical activity) was measured in OFT. Ethanol 70% was used to clean the apparatus after testing of each mouse.

2.3.3. Splash test

Self-care and motivational difficulties are investigated in rodents by using splash test. In this test, grooming activity in response to a sweet solution is considered as an indirect measure of palatable solution intake. A 10% sucrose solution was spurted on the dorsal coat of mice while they were in their home cages and mice were videotaped for 5 min. In this test, grooming activity behaviors include nose/face grooming, head washing, and body grooming (Ducottet et al., 2003; Marrocco et al., 2014; Haj-Mirzaian et al., 2016a).

2.3.4. Sucrose preference test (SPT)

The SPT was done to investigate the hedonic state in animals (Wallace et al., 2009; Amini-Khoei et al., 2015; Amiri et al., 2016a). In this test, two bottles of tap water were introduced to each mouse for the first two days. Then, for the second two days, a bottle containing 1% sucrose solution substituted one of the bottles. For sucrose preference test, animals were deprived of food and water for 5 h, and then SPT was carried out during 1 h using two bottles in each cage, one contained 1% sucrose solution and the other one was filled with tap water. SPT was measured using the following equation: sucrose preference = sucrose consumed/(sucrose consumed + tap water consumed).

2.4. Mitochondrial factors

Animals were sacrificed and hippocampi were separated on ice-cold surface and immediately snapped freeze in liquid nitrogen. Homogenization was done at 4 °C using cold mannitol solution containing 0.225 M D-mannitol, 75 mM sucrose, and 0.2 mM EDTA based on our previous studies and other documents (Lores-Arnaiz et al., 2010; Amiri et al., 2017). The homogenization was done in a glass homogenizer with a Teflon pestle and centrifuged at 1000 ×*g* for 10 min at 4 °C to remove the nuclei, unbroken cells, and other non-subcellular debris.

The supernatant was centrifuged at 10,000 ×*g* for 10 min as a source of hippocampal mitochondria. The heavy mitochondrial fraction was collected and re-suspended in the mannitol solution and, re-centrifuged twice at 10,000 ×*g* for 10 min. The resulting pellet (P_2 fraction), including both synaptic and non-synaptic mitochondria was re-suspended in desired buffer based on oxidative stress markers including ROS production, ATP, and glutathione (GSH) (Ahadpour et al., 2016). To keep the uniformity of experimental condition, the mitochondrial samples (100 μg protein/ml) were selected in all oxidative stress experiments. Mitochondrial protein concentration was determined by the Coomassie blue protein-binding method using BSA as the standard (Bradford, 1976).

2.4.1. Measurement of ROS formation

Mitochondrial ROS formation was measured using DCFH-DA reagent using HITACHI fluorescence spectrophotometer at excitation and emission wavelengths of 495 and 520 nm, respectively (Jafarian et al., 2013). Briefly, mitochondria (normalized to 100 μg of mitochondrial protein) were incubated in respiratory buffer containing 0.32 mM sucrose, 10 mM Tris, 20 mM MOPS, 0.05 mM EGTA, 0.5 mM MgCl2, 0.1 mM KH2PO4, and 5 mM sodium succinate and 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (final quantity of 10 μM). The fluorescence intensity was recorded in 5, 30 and 60 min intervals for 1 h and reported as a percent value based on the formula × 100:

$$\frac{\text{Fluorescence intensity in treated groups} - \text{Fluorescence intensity in saline group (mitochondria)}}{\text{Fluorescence intensity in saline group} - \text{Fluorescence intensity of DCF in media (without mitochondria)}}$$

2.4.2. ATP assay

For ATP assay, 0.5 ml of mitochondrial homogenate in TCA (6%) was pooled with 0.5 ml of KOH 0.05 M (on ice), then, supplemented with 1 ml deionized water; after 2 min, 650 μl of KH2PO4 (0.05 M) was added, and vortexed. After filtering, ATP level in each sample was determined using luciferase enzyme based on criteria described previously (Eskandari et al., 2012; Haj-Mirzaian et al., 2016a). Bioluminescence intensity was measured using Sirius tube luminometer (Berthold Detection System, Germany).

2.4.3. Glutathione (GSH) assay

Glutathione levels were measured according to the method described by Jayakumar et al. (Jayakumar et al., 2014), using 5, 5′-dithiobis- (2-nitrobenzoic acid) as an indicator. For this purpose, 0.1 ml of supernatant was added into 0.1 $mol \cdot L^{-1}$ of phosphate buffer and 0.04% DTNB in a total volume of 3.0 ml (pH 7.4). The generated color was measured at 412 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was considered as μg/mg protein.

2.5. Nitrite assay

Nitrite, as the nitric oxide (NO) end-product, was measured in our samples according to our previous studies (Amiri et al., 2015a, 2016b). Colorimetric assay based on the Griess reaction was used to assess the nitrite concentration. In brief, 100 μl of samples were mixed with 100 μl Griess reagent. Following 10 min incubation at room temperature, absorbance was measured at 540 nm in an automated plate reader. Concentration of nitrite was calculated by reference to a standard curve of sodium nitrite (Sigma, USA) and normalized to the mg protein of each sample.

2.6. Real-time RT-PCR analysis for hippocampus inflammatory genes

At first total RNA using TRIzol reagent (Invitrogen) was extracted from hippocampi. Alterations in the mRNA levels of genes were determined using qRT-PCR after the reverse transcription of 1 μg of RNA from each sample using PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). qRT-PCR was done on a light cycler device (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). Thermal cycling conditions were including an initial activation step for 30 s at 95 °C afterwards 45 cycles as well as a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was performed to certify whether all primers yielded a single PCR product. The genes and their primers are listed in Table 1. Histone H2A variant, *H2afz*, was used as a house-keeping gene (normalizer) and alterations in expression of each target mRNA in comparison with *H2afz* was measured based on $2^{-\Delta\Delta Ct}$ relative expression formula, as described in our previous publications (Amini-Khoei et al., 2016; Haj-Mirzaian et al., 2016b).

2.7. Statistics

The sample size was calculated by G power software (ver.3.1.7, Franz Faul, Universitat Kiel, Germany). We set α error at 0.05 and power (1-β) at 0.8 and the essential total sample size for each group was calculated as 6–8 in behavioral assessments and 3–6 in molecular experiments. In addition, we calculated the power value in each investigational group and have founded that the power values were >0.8 in all ANOVA analyses. Comparison between the groups was analyzed using *t*-test and one-way analysis of variance (ANOVA) followed by tukey's post hoc tests using the Graph-pad prism software (version 6). $P < 0.05$ was considered statistically significant.

3. Results

3.1. MS paradigm provoked depressive-like behaviors in adult male mice

Our results indicated that MS provoked depressive-like behaviors in adult male mice. *t*-test analysis revealed that the duration of immobility time in the FST was significantly increased in MS mice in comparison with control counterparts ($t = 6.576$, $df = 10$, $P < 0.001$, Fig. 1A). Further, in comparison with control mice, we showed that MS induced a significant reduction in grooming activity time in the splash test ($t = 9.703$, $df = 10$, $P < 0.001$, Fig. 1B). Moreover, in the SPT, MS significantly reduced the consumption of sucrose solution in MS mice when compared to controls ($t = 5.334$, $df = 10$, $P < 0.001$, Fig. 1C). In the OFT, *t-test* analysis showed that the total distance moved (horizontal activity) ($t = 9.488$, $df = 10$, $P < 0.001$, Fig.1D), and also the number of rearings (vertical activity) $t = 3.496$, $df = 12$, $P < 0.01$, Fig.1E) significantly increased in MS group in comparison to control group.

3.2. Oxytocin attenuated the depressive-like behaviors-induced by MS

Different doses of OT (0.5, 0.75, 1 and 1.25 μg/mouse in 1 μl, i.c.v.) were used to determine the effective dose of OT on depressive-like behaviors in both MS and control animals. One-way ANOVA analysis revealed that there are significant differences between different groups in the FST ($F(9, 51) = 27.40$, $P < 0.001$, Fig. 2A), splash test ($F(9, 57) = 95.63$, $P < 0.001$, Fig. 2B), SPT ($F(9, 51) = 35.63$, $P < 0.001$, Fig. 2C), horizontal activity of the OFT ($F(9, 61) = 48.59$, $P < 0.001$, Fig. 2D) and vertical activty of the OFT ($F(9, 61) = 19.59$, $P < 0.001$, Fig. 2E).

Results of post-test analysis showed that treatment with OT (1.25 μg/mouse in 1 μl) produced significant changes in depressive-like behaviors in control mice when compared to saline-treated control mice in the FST ($P < 0.001$), splash test ($P < 0.001$), and SPT ($P < 0.05$). However, other doses of OT had no significant effect on above-mentioned behavioral tests in control animals. In addition, OT (0.5, 0.75, 1 and 1.25 μg/mouse in 1 μl) made no significant changes in horizontal and vertical activities in the OFT in control mice ($P > 0.05$, Fig. 2D and E, respectively). Post-test analysis demonestrated that unlike doses of 0.5 and 0.75 μg/mouse in 1 μl, OT in doses of 1 and 1.25 μg/mouse in 1 μl significantly attenuated the depressive-like behaviors of MS mice in the FST ($P < 0.01$ and $P < 0.001$), splash test ($P < 0.05$ and $P < 0.01$), and SPT ($P < 0.05$ and $P < 0.01$). In the OFT, tukey's post-hoc analysis revealed that administration of OT (1.25 μg/mouse in 1 μl) to MS mice significantly reduced horizontal activity when compared to saline-treated MS mice ($P < 0.05$). However, MS mice showed no response to the OT effects in vertical activity in OFT ($P > 0.05$). Our results showed that unlike control mice, dose 1 μg/mouse in 1 μl in MS mice made significant changes in behavioral tests relevant to depression. Since dose 1.25 μg/mouse in 1 μl produced significant alterations in the behavior of both control and MS mice, we selected dose 1 μg/mouse in 1 μl for our molecular experiments. In order to confirm the antidepressant-like effect of OT, we used atosiban (ATO, 10 μg/mouse in 1 μl) alone and or in combination with OT (1 μg/mouse in 1 μl). One-way ANOVA analysis revealed that there are significant differences between groups in the FST ($F(7, 39) = 39.23$, $P < 0.001$, Fig. 3A), splash test ($F(7, 42) = 119.1$, $P < 0.001$, Fig. 3B), SPT ($F(7, 39) = 47.93$, $P < 0.001$, Fig. 3C), horizontal activity of the OFT ($F(7, 48) = 42.78$, $P < 0.001$, Fig. 3D) and vertical activty of the OFT ($F(7, 48) = 14.33$, $P < 0.001$, Fig. 3E). As shown in Fig. 3, administration of atosiban in control mice significantly increased the depressive-like behaviors in the FST ($P < 0.05$), splash test ($P < 0.05$) and SPT ($P < 0.05$). Furthermore, co-treated of atosiban with OT in control groups failed to produce significant changes in comparison with saline- treated control mice in the FST, SPT, splash test and OFT. Treating MS mice with atosiban produced any significant alterations in the aformentioned behavioral tests when compared to saline-treated MS mice. Tukey's analysis showed that co-administration of atosiban plus OT significantly increased the depressive-like behaviors of MS mice in the splash test ($P < 0.05$), and SPT ($P < 0.05$). In the OFT, post-hoc analysis revealed that co-administration of atosiban with OT to MS mice failed to produce significant changes in horizontal activity and vertical activity when compared to saline-treated MS mice.

Table 1
Primer sequences.

Primer name	Forward sequence	Reverse sequence
H2afz	TCATCGACACCTGAAATCTAGGA	AGGGGTGATACGCTTTACCTTTA
Tnf-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
Il-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
Myd88	ATCGCTGTTCTTGAACCCTCG	CTCACGGTCTAACAAGGCCAG
Nlrp3	ATCAACAGGCGAGACCTCTG	GTCCTCCTGGCATACCATAGA

3.3. Effects of MS and OT on GSH concentrations in the hippocampus

Tukey's analysis showed that GSH levels in the hippocampus of MS mice were significantly decreased when compared to control animals ($P < 0.01$, Table 2). Treating MS mice with OT (1 μg/mouse in 1 μl) significantly restored the GSH levels in comparison with saline-treated

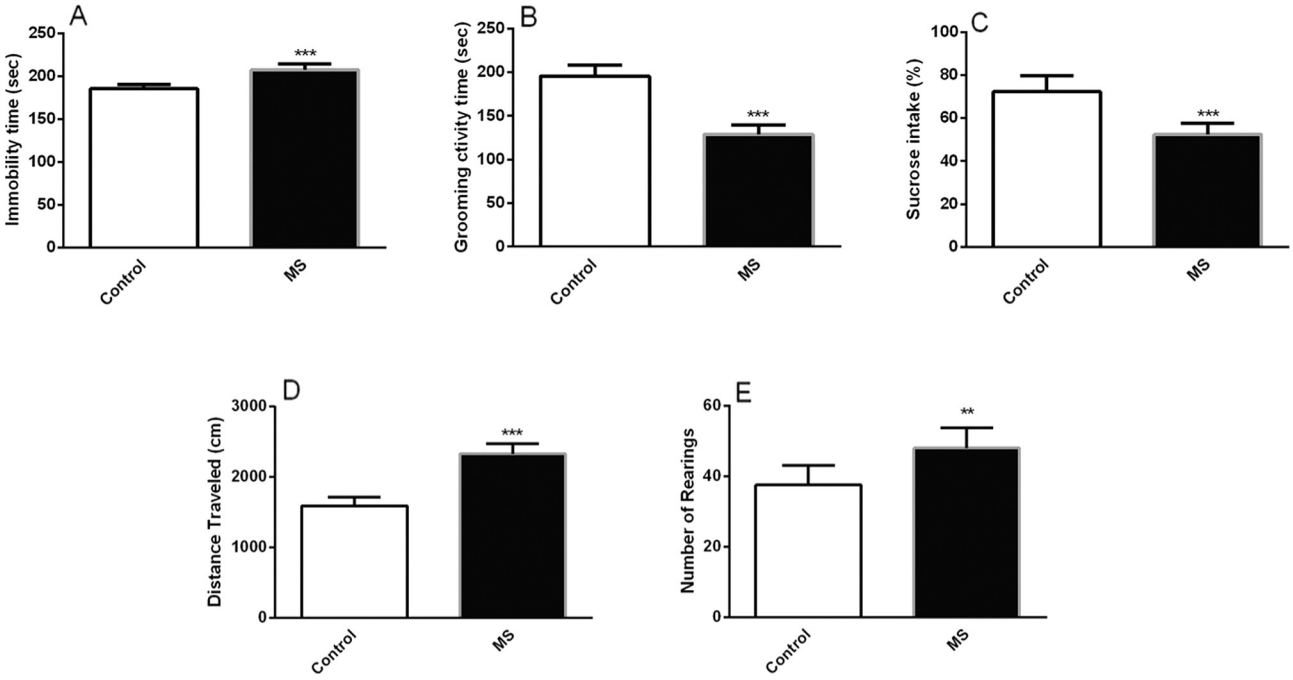

Fig. 1. Effects of MS on depressive-like behaviors in male mice: FST (A), splash test (B), SPT (C), OFT (D and E). Values are presented as the mean ± S.E.M from 6 to 8 animals and were analyzed using *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control mice.

MS mice ($P < 0.01$). In addition, treating control mice with atosiban/atosiban + OT significantly decreased the levels of GSH in comparison with saline-treated controls ($P < 0.01$). Atosiban alone or as co-administrated with OT failed to reverse the beneficial effects of OT on hippocampal GSH levels in MS mice. This result might be due to the dysfunction of OT receptors following MS paradigm. Recently, we have showed that MS led to dysfunction of OTergic system in the brain (Amini-Khoei et al., 2016).

3.4. Effects of MS and OT on ATP levels in the hippocampus

Our results showed that MS significantly reduced ATP levels in the hippocampus of MS mice in comparison with control mice ($P < 0.01$, Table.2). Treating MS mice with OT (1 μg/mouse in 1 μl) significantly increased ATP levels in the hippocampus of MS mice when compared to saline-treated MS mice ($P < 0.01$). Unlike MS group, administration of atosiban, as well as atosiban + OT in control animals significantly

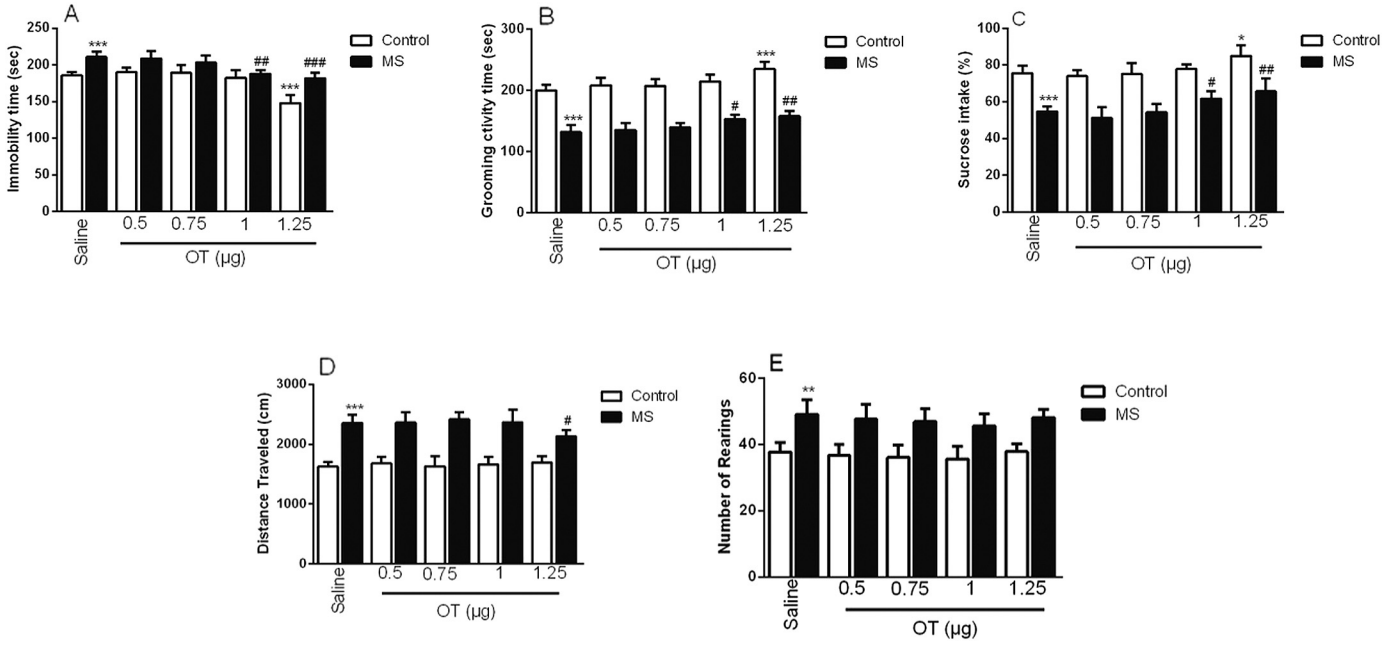

Fig. 2. Effects of several doses of oxytocin (0.5, 0.75, 1 and 1.25 μg/mouse in 1 μl, i.c.v.) on depressive-like behaviors in male mice: FST (A), splash test (B), SPT (C), and OFT (D and E). Values are showed as the mean ± S.E.M from 6 to 8 animals and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the saline-treated control mice, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the saline-treated MS mice. OT: oxytocin.

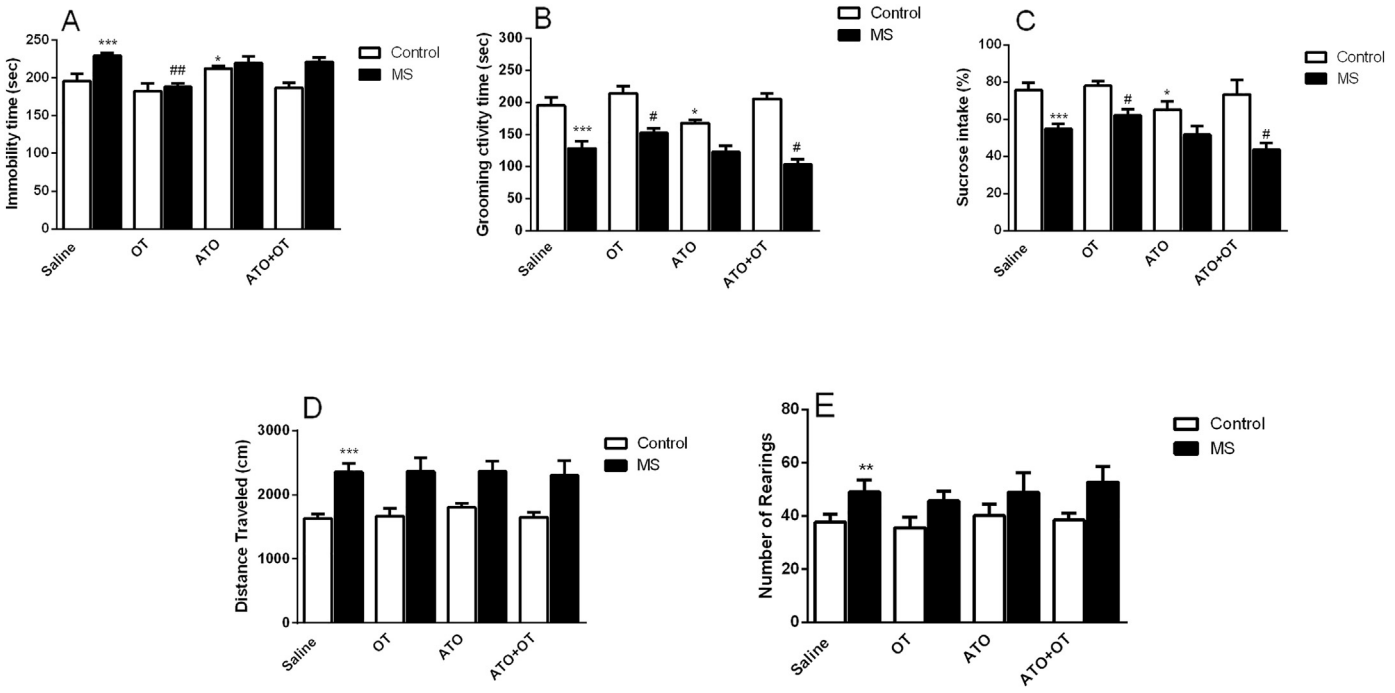

Fig. 3. Effects of co-administration of oxytocin (1 μg/mouse in 1 μl, i.c.v.) with atosiban (10 μg/mouse in 1 μl, i.c.v.) on depressive-like behaviors in male mice: FST (A), splash test (B), SPT (C), and OFT (D and E). Values are showed as the mean ± S.E.M from 6 to 8 animals and were analyzed using one-way ANOVA followed by Tukey's post hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the saline-treated control mice, #P < 0.05 and ##P < 0.01 compared with the saline-treated MS mice. OT: oxytocin and ATO: atosiban.

decreased the levels of ATP in comparison with saline- treated controls (P < 0.05 and P < 0.01, respectively).

3.5. Effects of MS and OT on nitrite levels in the hippocampus

Tukey's analysis determined that the nitrite level in the hippocampus of MS mice is greater than controls (P < 0.001, Table 2). In comparison with saline-treated MS mice, treatment with OT (1 μg/mouse in 1 μl) significantly decreased hippocampal nitrite levels in MS animals (P < 0.01).

3.6. Effects of MS and OT on ROS formation in the hippocampus

A significant increase in hippocampal ROS formation was observed in saline-treated MS mice at 3 time intervals when compared to saline-treated controls (P < 0.001 for all, Table 3). OT (1 μg/mouse in 1 μl) significantly decreased the elevated levels of ROS in the hippocampus of MS mice when compared with saline-treated MS animals (P < 0.001 for all). Furthermore, in comparison with saline-treated controls, administration of atosiban and also co-administration of atosiban with OT increased the hippocampal ROS levels (P < 0.001). Moreover, atosiban significantly enhanced ROS production in the hippocampus of MS mice in compared to MS controls at 5 min (P < 0.001) and 30 min (P < 0.001) intervals. Tukey's analysis showed that at 5 min interval co-administration of atosiban with OT significantly increased the levels of ROS production in comparison with saline-treated MS mice (P < 0.001).

3.7. Oxytocin decreased the expression of genes relevant to immune-inflammatory responses in the hippocampus

Our results on the effects of OT treatment on the expression of genes related to neuroinflammatory responses in the hippocampus are presented in Fig. 4. One-way ANOVA analysis showed that there are significant differences between experimental groups (F (39, 120) = 60.10, P < 0.001, Fig. 4). Tukey's analysis demonstrated the overexpression of

Table 2
Effects of MS and oxytocin on GSH, ATP, and nitrite levels in the hippocampus. Values expressed as mean ± S.E.M. (n = 3–6).

Groups	GSH (μg/mg protein)	ATP (nmol/mg protein)	Nitrite (nmol/mg protein)
Control	11.3 ± 0.7	10.1 ± 0.1	74 ± 4
Maternal separation (MS)	6.2 ± 0.15**	5.5 ± 0.2**	122 ± 7***
Control + oxytocin (1 μg)	11.4 ± 0.6	10.6 ± 0.2	78 ± 6
MS + oxytocin (1 μg)	10.4 ± 0.07##	9.9 ± 0.2##	97 ± 4##
Control + atosiban	7.2 ± 0.34**	8.1 ± 0.2*	70 ± 8
MS + atosiban	7.7 ± 0.7	5.5 ± 0.22	115 ± 6
Control + atosiban + oxytocin (1 μg)	6.3 ± 0.11**	6.1 ± 0.18**	81 ± 7
MS + atosiban + oxytocin (1 μg)	7.1 ± 0.19	7.6 ± 0.3	123 ± 4

*P < 0.05, **P < 0.01 and ***P < 0.001 compared with control group and ##P < 0.01compared with MS group.

Table 3
Effects of MS as well as oxytocin on percent of ROS formation in the hippocampus. Data represented as mean ± S.E.M. (n = 3–6).

Groups	DCF fluorescence intensity (%) 5 min	30 min	60 min
Control + saline	0	2.1 ± 0.05	3.4 ± 0.1
MS + saline	30.1 ± 0.8***	72.3 ± 2***	122 ± 3.5***
Control + oxytocin (1 μg)	0	1.8 ± 0.03	4.2 ± 0.2
MS + oxytocin (1 μg)	0.2 ± 0.01###	10.5 ± 0.01###	11.9 ± 0.5###
Control + atosiban	34.3 ± 1.7***	65.1 ± 1.3***	106.4 ± 7.4***
MS + atosiban	97.4 ± 3.6###	133.5 ± 4.8###	144.3 ± 5.5
Control + atosiban + oxytocin (1 μg)	54.3 ± 1.5***	60.3 ± 2.1***	124.5 ± 3***
MS + atosiban + oxytocin (1 μg)	52.3 ± 2.4##	63.4 ± 2.2	142.5 ± 3.7

***P < 0.001 compared with control group and ##P < 0.01 and ###P < 0.001 compared with MS+ saline group at the same time interval.

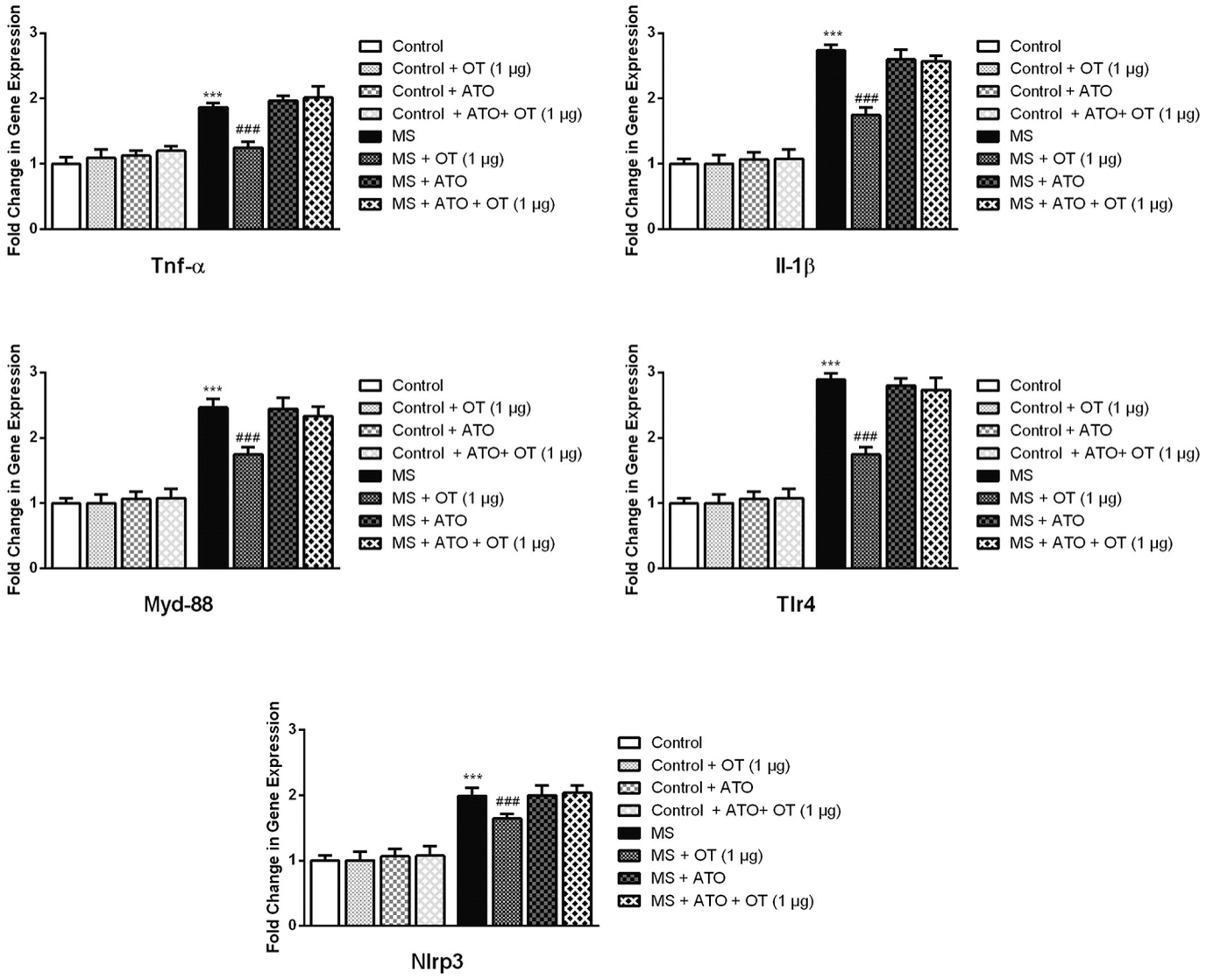

Fig. 4. Effect of oxytocin on hippocampal immune-inflammatory markers: Effect of intracerebroventricular saline and OT injections on *Tnf-α*, *Il-1β*, *Nlrp-3*, *Tlr4*, and *Myd88* gene expressions in the hippocampus of the animals, Data are expressed as the mean ± S.E.M and were analyzed by one-way ANOVA. ***P < 0.001 compared with the control group and ###P < 0.001 compared with MS group in each counterpart. OT:oxytocin, ATO: atosiban.

Tnf-α (P < 0.001), *Il-1ß* (P < 0.001), *Myd88* (P < 0.001), *Tlr4* (P < 0.001) and *Nlrp-3* (P < 0.001) in the hippocampus of MS mice in comparison with control animals. Our findings showed that following treatment with OT (1 μg/mouse in 1 μl), the expression of *Tnf-α*, *Il-1ß*, *Myd88*, *Tlr4* and *Nlrp-3* were significantly reduced in the hippocampus of MS mice when compared to saline-treated MS mice (P < 0.001 for all). However, OT failed to made significant differences in control mice when compared to saline-treated controls. In addition, administration of atosiban as well as co-administration of atosiban with OT did not exert significant changes in the expression of genes related to neuroinflammation when compared to their control counterparts.

4. Discussion

In the current study, we demonestrated that experiencing MS is capable of provoking depressive-like behaviors in adult male mice. These observed behavioral effects were associated with abnormal mitochondrial function and the increased expression of genes relevant to immune-inflammatory responses in the hippocampus. Further, we found that treating MS mice with OT significantly mitigated the negative effects of MS on hippocampus and behavior. Our results showed that OT has antidepressant properties against the effects of early life stress through improving mitochondrial function and reducing the neuroinflammation in the hipocampus.

Previous clinical and preclinical studies indicate that early life adversities have long-lasting endocrinological, behavioral and neurochemical effects on subjects. Since early relationship between infant and mother plays a critical role in shaping the offspring neurochemical system, MS as a stressful/traumatic event significantly increases the risk of mental disorders such as depression in adulthood (Kuhn and Schanberg, 1978; Kuhn et al., 1990; Matthews et al., 1996; Matthews and Robbins, 2003; Nemeroff and Vale, 2005). Previously reported results by our group have also shown that MS has negative effects on brain and behavior of animals by altering the seizure threshold, pain responses, depressive-like behaviors and neurotransmission such as dopaminergic system, opioid and OTergic systems (Amini-Khoei et al., 2015, 2016; Amiri et al., 2016a).

Previous studies have demonestrated that there are deficits in the open field activity of the animals which were chronically subjected to several stressors (Katz et al., 1981; Willner et al., 1987). It is important to note that there is an discrepancy between the results of different studies about OFT following MS paradigm (Shalev and Kafkafi, 2002; Cannizzaro et al., 2006; Millstein and Holmes, 2007). Our results showed that MS mice had increased vertical and horizontal activity in

the OFT. In this study, we conducted the OFT to ensure that locomotor activity of animals following MS or treatments does not affect the FST results, and the immobility of animals in the FST is not associated with their hypolocomotion. to (Amiri et al., 2016a). Also, our results showed that MS can provoke depressive-like behaviors in FST, SPT and splash test. FST is considered as a valid screening test for the evaluation of novel antidepressants. In this test, immobility time of rodents following experiencing the FST resemble to those passive behaviors of depressed patients following exposure to unpredictable and unscapable stressful conditions (Cryan and Holmes, 2005). In line with previous studies, our results showed that MS mice had higher immobility time in FST indicating the depressanteffect of MS on animals.

In addition to behavioral despair that we observed in FST results, anhedonia is another core symptom of depression that can be assessed by applying SPT to rodents (Katz and Sibel, 1982; Willner, 1985; Wallace et al., 2009). Our results showed a significant decrease in the cunsumption of sucrose indicating that MS is able to induce anhedonia in animals. Additionally, splash test is known to assess the self-care and motivational difficulties in rodents (Marrocco et al., 2014; Amiri et al., 2015b). We observed that MS mice exhibited low response to sucrose 10% (reduction in the grooming activity time) in the splash test suggesting the negative effects of MS on self-care and motivation behaviors.

Beyond serotonin hypothesis; role of inflammation, mitochondrial dysfunction and social factors have been considered as new theories for the pathogenesis od depression (Gardner and Boles, 2011; Maes, 2011; Slavich and Irwin, 2014). The elevated levels of pro-inflammatory cytokines has been reported in depressed patients (Maes et al., 1990, 2009; Blume et al., 2011). A study by Shelton and colleagues (Shelton et al., 2011) revealed an increase in the inflammatory cytokines in the frontal cortex of the brain of depressed patients which is involved in reward-related behavior.

Beyond the serotonin hypothesis, mitochondrial dysfunction and oxidative stress has been shown to be involved in the pathophysiology of depression (Tobe, 2013). In this regard, some studies have shown the involvement of mitochondrial genes (mtDNA and nDNA), respiratory chain enzyme ratios, and the rate of ATP production in the brain of depressed subjects (Gardner et al., 2003; Shao et al., 2008; Gardner and Boles, 2011). Chronic stress adversely affects the cellular energy metabolism through mitochondrial dysfunction (Picard et al., 2014). Brain is a highly active organ and mitochondria produce the ATP, which is necessary for cell survival and function. Increasing lines of research suggest that brain mitochondria not only are the first targets of stress, but also potently implicated in the pathophysiology of depression (Gardner and Boles, 2011; Morava and Kozicz, 2013). Recent evidence suggests the role of mitochondria in the production of damaged associated molecular patterns (DAMPs), which are released by the variety of cells (such as neurons, glial and immune cells) under stressful conditions (Krysko et al., 2011). Our results showed that abnormal mitochondrial function was associated with high level of oxidative and nitrosative stress (overproduction of ROS and NO), antioxidant impairment (decreased level of GSH) and impaired energy metabolism (decreased ATP production). These alterations in mitochondrial function are associated with the production of DAMPs under stressful conditions (Krysko et al., 2011). Since DAMPs are considered as endogenous ligands for the stimulation of TLR4, overexpression of these receptors along with main regulator of their signaling (MyD88 and TNF-α) suggests the involvement of TLR4 in the pathophysiology of depression (Hänsel et al., 2010; Liu et al., 2014). Considering the role of sterile inflammation in the pathophysiology of affective disorders, increasing lines of research have shown that mitochondrial dysfunction is associated with the activation of the NLRP3 that consequently instigates the expression of IL-1β (Anisman, 2009; Gurung et al., 2015). In agreement with above mentioned studies, we showed that applying MS paradigm significantly increased the expression of genes relevant to neuro-inflammatory response (*Il-1β*, *Myd88*, *Tnf-α*, *Tlr4* and *Nlrp3*).

The nine-amino-acid hypothalamic neuropeptide, OT, has a key role in the regulation of neurotransmission, psychogenic stress, anxiety, depression, social interaction, affiliative behaviors and emotion regulation (Heinrichs et al., 2003; Lim and Young, 2006; Hoge et al., 2008; Schulze et al., 2011). Focusing on the effects of OT on depression, Scantamburlo and colleagues (Scantamburlo et al., 2011) investigated the effects of intranasal OT as an adjunct to the antidepressant escitalopram in major depression, and showed a significant improvement in patients. Our result showed that administration of OT (i.c.v.) reveresed the effects of MS on behaviors related to depression. In this regard, OT treatment deceased the immobility time in the FST, Increased grooming activity time in the splash test, and also increased the sucrose consumption in the SPT. Our OFT results showed that OT decreased horizontal activity of MS mice. These results showed that acute administration of OT is capable of producing antidepressant-like effects in mice subjected to early life stress. These acute effects of OT suggest that early life stress is associated with an abnormal signaling of OTergic system. It has been shown that negative effects of MS are associated with epigenetic programming of stress on expression of some genes (e.g. oxytocin (*Oxt),* Oxytocin receptor *(Oxtr)*, and brain-derived neurotrofic factor or *Bdnf*). Thus, abnormal activity of OTergic system in MS mice maybe associated with the silencing of mentioned genes that administration of OT could acutely produce antidepressant effects (Roth et al., 2009; Kumsta et al., 2015).

Oxytocin regulates immune-inflammatory responses and has been shown to have antioxidant and anti-inflammatory activities. In this regard, inhibition of pro-inflammatory mediators such as TNF-α, IL-1β, and inducible nitric oxide synthase have been reported for OT (Düşünceli et al., 2008; Karelina et al., 2011; Akman et al., 2015; Yuan et al., 2016). A research by Yuan et al. (Yuan et al., 2016) demonstrated that OT has anti-neuroinflammatory effects and suggested that OT can be used as potential therapeutic agent for the alleviation of neuroinflammatory diseases associated with microglia activation. Further, another study showed that OT is able to improve antioxidant state and ameliorates oxidative injury (İşeri et al., 2005). In addition, another study demonstrated that OT can decrease the progression of atherosclerosis by decreasing NADPH-dependent superoxide activity and IL-6 release (Szeto et al., 2008). Also, protective effects of OT against free oxygen radicals, lipid peroxidation and also mitochondrial damage in the brain have been reported (Moosmann and Behl, 2002; Erbaş et al., 2012; Akman et al., 2015). In current study, we demonestrated that i.c.v. administration of OT attenuated the negative effects of MS on mitochondrial function and neuroinflammatory state in the hipocampus. Our findings showed that the expresion of genes related to neuroinflammation (*Il-1β*, *Myd88*, *Tnf-α*, *Tlr4* and *Nlrp3*) significantly decreased in OT-treated MS mice when compared to controls. Moreover, OT successfully improved mitochondrial function in MS mice by decreasing the ROS formation, increasing ATP levels and GSH levels. We also showed that OT decreased the NO levels in the hipocampus of MS animals. These results showed that OT has acute antidepressant-like effects in adult male mice exposed to early life stress, and its effects are partly associated with improvement of mitochondrial function and decreasing the inflammatory factors in the hippocampus. Considering the safety and rapid action of OT, further research is needed to clarify the potential application of OT could as a clinical antidepressant in humans or atleast as an adjuvant therapy to antidepressant for the treatment of depression.

5. Conclusions

In conclusion, results of this study showed that: 1) Maternal separation, as an early life challenge, provoked depresive-like behaviors in adult male mice, 2) adverse effects of MS were associated with abnormal mitochondrial function and immune-inflammatory responses in the hipocampus, 3) OT acutely mitigated the effects of MS on depressive-like behaviors in male mice, 4) Beneficial effects of OT against the adverse effects of MS are partly associated with the

improvement of mitochondrial function and attenuation of neuro-inflammatory responses in the hipocampus.

Acknowledgment

This study was supported by a research grant (NO: 94-04-30-31430) from Tehran University of Medical Sciences, Tehran, Iran. The authors would like to thank Dr. Hamid Reza Banafshe and Dr. Azam Mesdaghinia for their collaboration on this study.

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